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Synthesis of the heat shock protein, hsp70, appears to be essential for recovery from heat and chemical stress. Both because of the role of this protein in cellular recovery from stress and because of the possibility of using levels of hsp70 synthesis or accumulation as a measurement of cellular response in stress, it is important to study the stability of hsp70. We have shown that Drosophila hsp70 decays *in vitro* by an autoproteolytic mechanism (Mitchell et al., 1985). Autoproteolytic decay could be part of the feedback mechanism regulating the levels of hsp70 accumulation if it occurs *in vivo*. To determine whether autoproteolytic decay is occurring in vivo, we propose to identify the *in vivo* breakdown products of hsp70 and to compare their N-terminal sequences to those of the *in vitro* breakdown products. Precisely the same cutting site would indicate that the same protease may be responsible for the decay in both cases. We will also determine the site of the protease activity in hsp70 for the *in vitro* decay.

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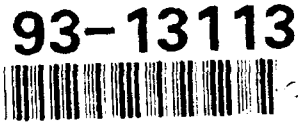

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AFOSR Grant No. F49620-92-J-0234

USAF Cellular Mechanism of Turnover of the Stress Induced Protein HSP70.

Annual Technical Report: 15 Apr. 92 - 14 Apr. 93

Summary:

Synthesis of the heat shock protein, hsp70, appears to be essential for recovery from heat and chemical stress. Both because of the role of this protein in cellular recovery from stress and because of the possibility of using levels of hsp70 synthesis or accumulation as a measurement of cellular response to stress, it is important to study the stability of hsp70.

We have shown that Drosophila hsp70 decays *in vitro* by an autoproteolytic mechanism (Mitchell et al., 1985). Autoproteolytic decay could be part of the feedback mechanism regulating the levels of hsp70 accumulation if it occurs *in vivo*. To determine whether autoproteolytic decay is occurring *in vivo*, we propose to identify the *in vivo* breakdown products of hsp70 and to compare their N-terminal sequences to those of the *in vitro* breakdown products. Precisely the same cutting site would indicate that the same protease may be responsible for the decay in both cases. We will also determine the site of the protease activity in hsp70 for the *in vitro* decay.

Autoproteolytic decay is part of the normal processing which occurs in many viral proteins and leads to the production of functional proteins. Perhaps breakdown products of hsp70 also have a function in the cell. In any case, if one of them is more stable than hsp70 itself, it may be more useful than intact hsp70 as a measure of cellular exposure to stress. An initial step toward looking at these possibilities is to identify the *in vivo* breakdown products of hsp70 and look at the stability of both hsp70 and its decay products *in vivo*. We propose to do this in Drosophila larvae and rainbow trout tissues.

During the tenure of this grant we propose to:
1) Identify the *in vivo* breakdown products of Drosophila and rainbow trout hsp70.

2) Sequence the N-termini of both the *in vivo* and the *in vitro* decay products of Drosophila and rainbow trout hsp70.
3) Identify the sites of proteolytic activity in both rainbow trout and Drosophila hsp70.
4) Use antibodies to hsp70 to look in detail at the kinetics of synthesis and decay of hsp70 and its breakdown products in Drosophila larvae and rainbow trout tissues.

Personnel:

Nancy S. Petersen	P. I.
Pat Young	Research Associate
Jeanne Willliams	Graduate Student
Ronald Bromley	Graduate Student
Frank Zgaynor	Undergraduate, lab assist.
	Undergraduate, fly care

Jeanne Williams is the graduate student who has been supported for the past year on this grant. She is one of the best graduate students we have had at the University of Wyoming in the almost ten years that I have been here. Besides doing the initial characterization of the rainbow trout heat shock response and cloning of the rainbow trout hsp70 gene, she has undertaken collaborative experiments with Harold Bergman (another AFOSR grant recipient at UW) to evaluate the levels of hsp70 found in rainbow trout tissues under controlled exposure to heavy metals. During the past year we have recruited another graduate student, Ron Bromley. Ron has subcloned the region of hsp70 which we expect to include the autoprotease and or the protease sensitive site. He plans to identify the type of protease involved in the in vitro decay and to identify the active site of the protease using this construct. Ron has been supported by the Molecular Biology Department this year, and we hope that he will be able to get continued support on the Air Force EPSCoR program. If the EPSCoR grant is funded we will also be able to recruit two more undergraduates to participate directly in the research projects.

Progress Report:

During the past year we have concentrated our efforts on characterizing the rainbow trout heat shock response and on generating antibodies specific for Drosophila hsp70 breakdown products. We have also used commercially available antibodies to tentatively identify hsp70 breakdown products in Drosophila, trout, chick and mouse, and we have looked in more detail at the kinetics of hsp70 breakdown in Drosophila.

Rainbow trout heat shock response:

The heat shock response has been characterized in rainbow trout liver and gill tissue. These tissues were dissected, heated, and labeled with ^{35}S-methionine immediately after killing the fish. The labeled proteins were visualized on autoradiograms of SDS-polyacrylamide gels. The increased synthesis of heat shock proteins is first detected at 20°C. The maximum synthesis of hsp70 occurs at 26°C in both tissues, with protein synthesis being completely inhibited by 30°C. The conditions for induction of hsp70 were very similar in all of the tissues examined (gill, liver, kidney. stomach, and intestine).

Messenger RNA was isolated from liver tissue which was kept at 10°C or at 26°C in MEM tissue culture media (Gibco) for an hour before freezing and mRNA preparation. PCR on reverse transcribed mRNA shows one mRNA band present in both control and heat shocked samples with an extra faint band appearing in the heat shocked sample. PCR primers were designed to conserved regions at the N-terminus and C-terminus of the coding region and amplify DNA of the predicted size of 2kb. The major PCR product has been cloned in to bluescript and several clones are ready for sequencing. The relative amounts of hsp70 RNA cannot be determined from these PCR experiments since they were not done under conditions where the mRNA was the limiting component of the system. Northern blots are currently being done to determine the number, size, and relative abundance of the hsp70 messages in these tissues. This is to determine whether hsp70 mRNA accumulates in response to heat shock as it does in most other systems, and to confirm that the PCR product we have cloned represents the major heat inducible mRNA in rainbow trout.

Antibodies to Drosophila hsp70 fragments:

We have subcloned three different fragments of hsp70 into the PGEX expression vectors. These fragments represent the conserved amino terminus of the protein, the carboxy-terminus of the protein, and an internal sequence (the Ava1 - BamH1 fragment) which has been implicated in autoproteolysis. Each fragment was expressed in E. coli as a glutathione S-transferase fusion protein. The fusion proteins purified by binding to glutathione sepharose beads. Purification of the hsp70 fragments was done by thrombin digestion at a thrombin site engineered at the junction between the two sequences.

Antibodies to each fragment are being made in rat by Coalico Biologicals Inc., Reamstown, PA, and should be available for testing in the first part of June.

Turnover of hsp70 in Drosophila tissues:

Our earlier experiments and those of Karen Palter and Elizabeth Craig had shown that hsp70 in whole larvae and adult flies has a half life of 3-5 hours. We have repeated these experiments using individual tissues from larvae and pupae rather than whole animals. We wanted to see if there were major differences in the half life of hsp70 in tissues and whether the the breakdown was a cellular event or whether it required interaction of tissues in the whole animal. Brains and salivary glands were dissected from 3rd instar larvae. Wings were dissected from pupae of different ages. These were labeled with 35S-methionine during a mild heat shock of 35° for 30 min., and chased for 2- 6 hours at 25°C. Hsp70 decays with a half life of 3-4 hours in different larval and pupal tissues. The half life of hsp70 in wings was the same at different developmental stages. These results indicate that the turnover of hsp70 occurs at the cellular level and is similar in the different tissues and at the different times which were examined. An initial experiment on the stability of hsc70 indicates that it is somewhat more stable than hsp70.

One experiment to look at the half life of hsp70 in trout liver indicated that trout hsp70 is more stable than Drosophila as it did not snow significant decay in 24 hours.

Use of antibodies to detect changes in hsc70 (heat shock cognate)

levels in trout tissues after exposure to metal contaminated water:

Rainbow trout were exposed for three weeks to clean water or water with two times the EPA allowable concentrations of Zn++, Cu++, Cd++, and Pb++ (100µg/lZn++, 24µg/l Cu++, 2.2µg/l Cd++, and 6.4µg/l Pb++). The fish were killed with MS-222 (an anesthetic which does not induce the heat shock response). Tissues (gill, liver, kidney, stomach) were labeled with ^{35}S-methionine. In all 24 adult rainbow trout, and 24 juvenile trout were examined. Proteins were extracted from each sample and run on SDS-PAGE gels. The gels were blotted to PVDF paper and StressGen 72/73 antibody to hsp70 was used to detect levels of hsp70 related proteins. Blotting conditions were worked out so that the staining of hsc70 increased linearly with protein concentration. There were very different ^{35}S-methionine labeling patterns for the different tissues, however exposure to metal contaminated water did not induce measurable increased in hsc70 synthesis or synthesis of any other major proteins seen on these autoradiograms. The levels of hsc70 (identified as hsc70 since levels didn't increase significantly with heat shock) in the different tissues were quite variable, but were not significantly different between controls and exposed fish with one exception. Juvenile fish gills from fish raised in 2X water have at least two times the amount of hsc70 as the controls. One problem we encountered was that the Stress-Gen antibody reacted only weakly with the rainbow trout tissues as compared to mouse and chick tissues. This could be due to lower levels of hsc70 in fish or to lower affinity of the antibody for fish hsc70. We hope to be able to obtain a more specific antibody using the fish hsp70 gene expressed in E. coli.

Progress on specific aims:

The specific aims have been changed from the original grant by replacing characterization of human hsp70 turnover with characterization of rainbow trout hsp70 turnover. This has resulted in a change in the overall time required to complete the project because the cloned human hsp70 gene is available while the rainbow trout heat shock response is incompletely described in the literature and only partial sequences of the rainbow trout hsp70 gene are available.

1) Identification of the in vivo breakdown products of Drosophila and rainbow trout hsp70:

Breakdown products of hsp70 have tentatively been identified in tissues from rainbow trout, Drosophila, mouse, and chicken. We have used three commercially available monoclonal antibodies to look for potential breakdown products of hsp70 or hsc70 in heat shocked trout liver, mouse liver, chicken liver, and Drosophila salivary glands. The antibodies used were StressGen SPA-820 and Affinity Bioreagents MA3-006 and MA3-007.

All three antibodies reacted strongly with a 70kD band in mouse and chick. In mouse there was also a major cross reacting band at 46kD (the approximate size expected for the breakdown product). In chicken cross reacting bands at 44kD and 36kD were observed. The Affinity Bioreagents antibodies reacted weakly to Drosophila and fish 70kD proteins. In Drosophila, MA3-007 (to a conserved epitope in the N-terminus of the protein) reacted with a band at 43kD, indicating that there may be a 43kD breakdown product in Drosophila salivary glands, and that if so, it is derived from the N-terminal region of the protein.

The StressGen antibody reacted weakly with trout and not at all with Drosophila hsp/hsc 70. Putative breakdown products in trout have molecular weights of 36kD and 34kD. The amount of each of these varies in different tissues.

Positive identification of these as breakdown products will be obtained by N-terminal sequencing.

2) Sequencing of the N-termini of both the in vivo and the in vitro decay products of Drosophila and rainbow trout hsp70:

This project requires antibodies specific for different fragments of hsp70. Progress generating the antibodies is described above.

3) Identification of the sites of proteolytic activity in both rainbow trout and Drosophila hsp70:

The region implicated as the auto protease in Drosophila has been subcloned into PGEX.2T. This construct will be used with different protease inhibitors to determine the type of protease and the active site for the activity.

For rainbow trout, this project awaits the sequencing and subcloning of the rainbow trout hsp70 gene.

4) Use of antibodies to hsp70 to look in detail at the kinetics of synthesis and decay of hsp70 and its breakdown products in Drosophila larvae and rainbow trout tissues:

Initial experiments have been done in Drosophila tissues and are described above. With the more specific antibodies we will have in June we will be able to look at the accumulation of breakdown products in these tissues.

Publications

Abstracts:

(1992) Petersen, N. S., Williams, J., and Young, P. Hsp70 is an Unstable Protein in Drosophila Tissues and Cell Lines. Molecular Biology of the Cell 3; s1031.

(1992) Petersen, N.S., Williams, J., and Young, P. Rapid Turnover of hsp70 in Drosophila larval and pupal tissues. ASCB Molecular Chaperonins Meeting Abstracts.

(1992) Petersen, N. S., Williams, J., and Young, P. Hsp70 Stability., SETAC News, in press.

Papers in preparation:

Williams, J., Young, P., Farag, I., Bergman, H., and Petersen, N. Increased levels of hsp70 in juvenile rainbow trout gills exposed to heavy metals.

Petersen, N., Young, P., and Williams, J. Hsp70 and hsc70 turnover rapidly in Drosophila tissues.